Proteomics

Human Diseases and Advances

Steven Tiff

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Preface

Proteomics is a diverse field dealing in study of various proteins and their structure and functions. Biomedical research has reached the stage where a novel era of identifying a disease or a protein globally has emerged. During the post-genomic era, proteomics played an extremely significant role in analyzing molecular functions of proteins and determining biomarkers in diseases affecting human beings. Mass spectrometry, high-density antibody and protein collection, and 2D gel electrophoresis are few of the generally employed techniques in proteomics field. This book elucidates four significant and varied areas of present proteomic research: proteomic analysis of protein functions, organelles and secretome proteomics, proteomic discovery of disease biomarkers, and proteomic techniques for analyzing disease procedures. The use of these techniques in analyzing proteins in different diseases has now become a significant part of biomedical research across the globe. This book will serve as a useful source of reference for students, researchers as well as clinicians.

This book is the end result of constructive efforts and intensive research done by experts in this field. The aim of this book is to enlighten the readers with recent information in this area of research. The information provided in this profound book would serve as a valuable reference to students and researchers in this field.

At the end, I would like to thank all the authors for devoting their precious time and providing their valuable contribution to this book. I would also like to express my gratitude to my fellow colleagues who encouraged me throughout the process.

Editor

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Part 1

Proteomic Discovery of Disease Biomarkers

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Overview of Current Proteomic Approaches for Discovery of Vascular Biomarkers of Atherosclerosis

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1. Introduction

Cardiovascular diseases are the leading cause of mortality and morbidity in developed countries being atherosclerosis the major contributor. Atherosclerosis is a form of chronic inflammation characterized by the accumulation of lipids and fibrous elements in medium and large arteries (Libby, 2002). The retention of apoB-100 containing lipoproteins (mainly LDL and Lp(a)) in the subendothelial space and their subsequent oxidation is thought to be the leading event in the development of atherosclerotic lesions (Williams & Tabas, 1995). The degree of inflammation, proteolysis, calcification and neovascularization affects the stability of advanced lesions. Plaque rupture and thrombosis are the most important clinical complications in the pathogenesis of stroke, coronary arteries and peripheral vascular diseases (Lutgens et al., 2003). So, the identification of early biomarkers of plaque presence and susceptibility to ulceration could be of primary importance in preventing such a life-threatening event. Disease aetiology is very complex and includes several important environmental and genetic risk factors such as hyperlipidemia, diabetes, and hypertension. In this regard elevated plasma levels of LDL cholesterol and low levels of HDL cholesterol have been long associated with the onset and development of atherosclerotic lesions. Although enormous efforts have been done to elucidate the molecular mechanisms underlying plaque formation and progression, they are not yet completely understood. In the last years, proteomic studies have been undertaken to both elucidate pathways of atherosclerotic degeneration and individuate new circulating markers to be utilized either as early diagnostic traits or as targets for new drug therapies.
This chapter will provide an overview of latest advances in proteomic studies on atherosclerosis and some related diseases, with particular emphasis on vascular tissue proteomics and lipoproteomics.

2. Application of proteomic technologies to the study of atherosclerosis

Atherosclerosis is a very complex pathology in terms of cell types involved, inflammatory mechanisms and multifactorial aetiology. Many efforts have been done to shed light on the mechanisms underlying atherogenesis and to identify new circulating biomarkers which, along with traditional risk factors, will help in early diagnosis and prevention as well as in

monitoring the effects of pharmacological agents. To address these issues, proteomic studies have been focused on different matrices such as vascular cell/tissues, looking at both proteomes and secretomes, plasma/serum, urine, and purified plasma lipoprotein fractions (fig. 1).

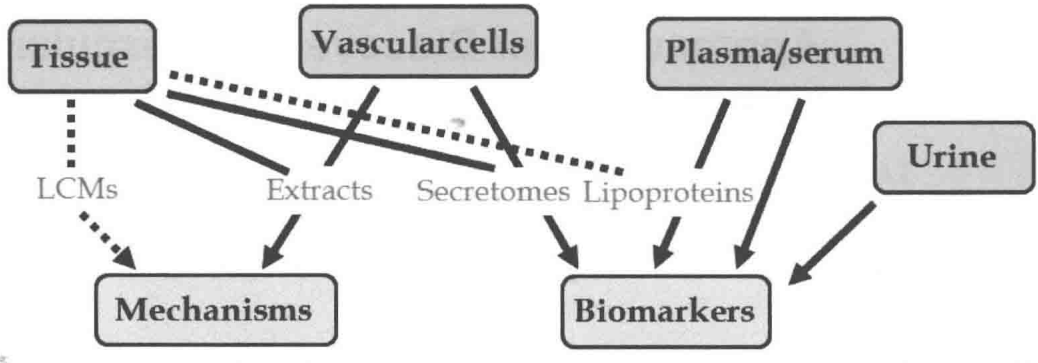

Fig. 1. Overview of the main targets of proteomic studies searching for both mechanisms of atherogenesis and biomarkers of atherosclerotic lesion presence and progression. Dotted lines represent almost unexplored paths. LCMs, laser-captured microdissections

To date, several proteomic approaches, such as 1D-2D electrophoresis (1DE-2DE) followed by mass spectrometry (MS) analyses, western arrays, protein arrays, and gel-free MS based proteomics, have been applied in the search of vascular biomarkers of atherosclerosis. Often, classical biochemical methods, mainly western blotting (WB), ELISA, and immunohistochemistry (IH) have been used to validate the proteomic results.

2.1 Vascular tissue proteomics

Even though tissue analyses frequently provide useful data, there are major drawbacks in analysing human atherosclerotic specimens. Atherosclerotic plaques are quite complex in terms of vascular cells and extracellular components. In this respect, besides vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), they are composed of inflammatory cells, filtered plasma proteins, new-formed extracellular matrix, cellular debris and end-products of lipid and protein oxidation. Another critical point in the *in situ* analysis of protein expression within atherosclerotic plaques is the choice of the appropriate control. It would be desirable to utilize control specimens from the same vascular district of the same patient, in order to minimize intrinsic tissue differences, and from surgical endarterectomy rather than from post-mortem material, to avoid the occurrence of proteolytic modifications prior to analysis. Also the availability of a significant number of human specimens could be limiting. Because of the complexity of advanced lesions (Stary, 2000; Virmani et al., 2000) in terms of necrotic core dimension, fibrous cap thickness, inflammatory and proteolytic components, careful histochemical classification is needed. Moreover, results from different advanced lesion typologies are difficult to interpret because they could be either associated to the lesion development or merely a consequence of the advanced condition. In the latest years proteomic technologies have been applied to human diseased tissues to both characterize mechanisms of advanced atherosclerotic plaque development, mainly those responsible for its instability, and to identify markers useful in

diagnosis and patients treatment. Compared to tissue specimens of human origin, animal models, mainly rodents, have been utilized to study the mechanisms underlying the early stages of lesion formation.

2.1.1 Studies on animal models

Apolipoprotein E-deficient mouse is the most popular murine model in cardiovascular research and has revealed important insights into mechanisms affecting atherogenesis. Mayr et al. analysed aortic lesions from apolipoprotein $E^{-/-}$ and wild type mice classified as light, medium, and severe according to lesion-covered areas on the aortic surface (Mayr et al., 2005). As expected, authors found an increase of inflammatory cells, a decrease of VSMCs, and an accumulation of serum proteins associated to an impaired endothelial barrier function with lesion progression. Interestingly, immunoglobulins, that were barely detectable in apolipoprotein $E^{+/+}$ mice, accumulated even in aortas of young apolipoprotein $E^{-/-}$ mice. The authors identified 79 differentially expressed spots. Moreover, they suggested an increase in oxidative stress with lesion progression evaluating the ratio between the oxidized and the reduced forms of peroxiredoxin, the former resulting in a charge shift toward a more acidic isoelectric point. Overall, they found a linear relationship between the degree of peroxiredoxin-Cys oxidation and the extent of lesion formation in aortas of apolipoprotein E-deficient mice. Almofti et al. applied 2DE coupled to matrix-assisted laser desorption/ionization time of flight (MALDI TOF) MS analysis to a rat model of atherosclerosis. They induced atherosclerosis by a single dose of vitamin D3 associated with a high fat diet and identified 46 proteins differently expressed in diseased tissues. Among them, 18 proteins, including a group of oxidization-related enzymes, were found to be up-regulated, while 28 proteins were found down-regulated (Almofti et al., 2006). Vascular endothelium plays important physiological roles in vascular homeostasis, coagulation, inflammation, as well as tissue growth and repair. Impairment of the endothelial function is an early event in atherosclerotic lesion formation leading to overexpression of adhesion molecules as well as secretion of pro-inflammatory and chemotactic cytokines. An affinity-based proteomic approach was used by Wu et al. (Wu et al., 2007) to identify vascular endothelial surface proteins differentially expressed in aortic tissues of apolipoprotein E deficient mice. After *in situ* perfusion of vascular bed with a solution containing a biotin-derivative, biotinylated endothelial proteins were extracted, purified by affinity enrichment with streptavidin-agarose beads, and resolved by SDS-PAGE. The whole gel lanes were cut into slices that were subjected to tryptic digestion for nano liquid chromatography (LC) MS/MS analysis. In this way, 454 proteins, mainly extracellular or associated to cell membrane, were identified. Among them, there were cell adhesion molecules, accounting for the largest category, followed by proteins involved in signal transduction and transport. Interestingly, proteins associated with immune and inflammatory responses were more than doubled in atherosclerotic aorta (13%) in comparison to normal aorta (6%). On the other hand, proteins involved in lipid metabolism were decreased by 34% in atherosclerotic aorta. A rat model has been recently used for a proteomic study on the effects of blood shear stress on atherogenesis (Qi et al., 2008). It is well known that blood shear stress affects endothelial cell shape and orientation, as well as vascular wall permeability. Indeed, regions of arterial branching or curvature, where blood flow is not uniform, are preferential sites for lesion formation. By comparing homogenates of aortas kept under two levels of shear stress in a perfusion culture system for 24 hours, Qi et al. detected a reduced expression of protein Rho-GDP dissociation inhibitor alpha (Rho-GDIα) in low shear stress conditions and

demonstrated, by siRNA technology, that this reduction enhances VSMC migration and apoptosis.

2.1.2 Studies on human tissues

As from 2003, 14 researches on human atherosclerotic plaque proteomics have been published; the diseased tissues used as matrices were coronary arteries (2/14), carotid arteries (11/14), and aortas (1/14).

Most of them were conducted by using two-dimensional electrophoresis coupled to mass spectrometry as analytical method (10/14). The sample source, the methodology applied, and the most relevant findings of these studies are summarized in table 1.

In 2003, You et al., by analysing 10 diseased (coronary artery disease, CAD) and 7 normal autoptic coronary arteries, reported about 2 fold increase of the ferritin light chain in the pathological specimens (You et al., 2003). Quantitative analysis by real-time PCR showed a decrease in ferritin light chain mRNA expression in diseased tissues suggesting that the increased expression of ferritin light chain in CAD coronary arteries may be related to increased protein stability. This result highlights the importance of protein expression analysis in studying disease-associated gene expression. Donners et al. analysed 5 stable plaques and 6 lesions with a thrombus from patients undergoing carotid endarterectomy, classified according to Virmani et al. (Donners et al., 2005). By 2DE analysis, they identified vinexin-β and α1-antitrypsin as differentially expressed. However, neither immunohistochemistry nor western blotting confirmed vinexin-β differential expression underlining the importance of validating proteomic results by other biochemical methods. Conversely, western blotting of 2D gels revealed, in lesions with a thrombus, the expression of six isoforms of the acute phase protein α1-antitrypsin, one of which was uniquely expressed in thrombus-containing plaques. Sung et al. analysed non-diseased and atherosclerotic specimens from 7 patients undergoing aorta bypass surgery. They identified a panel of 27 proteins differentially expressed in the atherosclerotic aorta involved in a number of biological processes, including calcium-mediated processes, migration of VSMCs, matrix metalloproteinase activation and regulation of pro-inflammatory cytokines (Sung et al., 2006). A different approach was adopted by Martin-Ventura et al. who analysed the protein secretion profiles obtained from 35 cultured atherosclerotic plaques (10 femoral, 25 carotids) and 36 control arteries (24 mammary, 12 radial) in the search of new biological markers potentially released by the arterial wall into the plasma (Duran et al., 2003; Martin-Ventura et al., 2004). In particular, they isolated and analysed the secretomes from non-complicated and ruptured/thrombosed areas of the same cultured carotid plaque so avoiding the variability of the control specimens. They showed that, compared to control arteries, heat shock protein 27 (HSP27) secretion into the culture medium was significantly lower in atherosclerotic plaques and barely detectable in complicated plaque supernatants, as confirmed by WB analysis. They also evidenced a 20-fold reduction in HSP27 levels in the plasma of patients with carotid stenosis respect to healthy controls so identifying HSP27 as a possible marker of atherosclerosis. The same research group evaluated the effects of incubation with atorvastatin, a 3-hydroxy-3-methylglutaryl CoenzymeA reductase inhibitor, on the secretomes of cultured atherosclerotic plaques (Durán et al., 2007). They identified 24 proteins that were increased and 20 proteins that were decreased in atherosclerotic plaque supernatants compared to controls. Interestingly, the presence of atorvastatin in culture medium reverted secretion of 66% proteins to control values. In this report, authors identified cathepsin D as a potential target for therapeutical treatment of atherosclerosis.

Human tissues (Methods)	Results	Known functions	Ref.
10 coronary arteries from CAD patients vs 7 normal autoptic coronary arteries (2DE of homogenates, LC-MS/MS, WB, rt-PCR)	↑ ferritin light chain ↓ ferritin light chain mRNA	modulation of oxidation	You et al., 2003
6 carotid plaques containing a thrombus vs 5 advanced stable lesions (2DE of homogenates, MALDI-TOF/TOF MS, LC-MS/MS, WB, IH)	↑ α1-antitrypsin	acute-phase protein	Donners et al., 2005
7 atherosclerotic aortic specimens vs biopsies of the normal tissue from the same patients (2DE of homogenates, MALDI-TOF MS, WB)	↑ 39 proteins (27 identified)	signal transduction angiogenesis MMP activation regulation of pro-inflammatory cytokines	Sung et al., 2006
35 atherosclerotic endarterectomies (10 femoral, 25 carotids) vs 36 control endarteries (24 mammary, 12 radial) (2DE of secretomes, MALDI-TOF MS, LC-MS/MS and IMAC combined with MALDI Q-TOF MS/MS, WB, ELISA, IH)	↓↓ HSP27 secretion ↓↓ HSP27 plasma levels	anti-inflammatory down-regulation of the apoptotic signaling pathway	Duran et al., 2003; Martin-Ventura et al., 2004
21 stenosing complicated carotid regions with/without atorvastatin treatement vs fibrous regions (ex vivo) (2DE of secretomes, MALDI-TOF MS, LC MS/MS, WB)	↑ 24 proteins ↓ 20 proteins Treatment reverts the differential protein secretion	modulation of oxidation and inflammation structural signaling pathway cholesterol metabolism	Durán et al., 2007
29 unstable carotid plaques vs 19 stable carotid plaques (2DE of extracts, MALDI-TOF MS, WB)	↑ ferritin light subunit ↑ superoxide dismutase 2 ↑ fibrinogen fragment D ↓ superoxide dismutase 3 ↓ glutathione S-transferase ↓ Rho GDP-dissociation inhibitor 1 ↓ annexin A10 ↓ HSP 20 ↓ HSP 27	modulation of inflammation and oxidative stress	Lepedda et al., 2009
10 complicated segments in the internal carotid artery (ICA) vs 10 stable segments in the common carotid artery (CCA) (2-D DIGE of homogenates, LC-MS/MS, IH)	↑ 6 proteins ↓ 11 proteins 2 proteins with isoform dependent distributions	signal transduction transport cell growth metabolism	Olson et al., 2010

Human tissues (Methods)	Results	Known functions	Ref.
10 carotid plaques vs reference synthetic gel (2DE of homogenates, LC-MS/MS)	Identification of proteins exclusive to plaque		Terzuoli et al., 2007; Porcelli et al., 2010)

IMAC, immobilized metal affinity chromatography. ↑, increase. ↓, decrease.

Table 1. 2DE coupled to MS studies on the human atherosclerotic plaque.

Since carotid plaque rupture and thrombosis are the most important clinical complications in the pathogenesis of cerebro-vascular diseases, many efforts have been done to elucidate mechanisms underlying plaque vulnerability and to identify reliable specific markers of plaques prone to rupture. In a previous study we provided evidence for a wide fragmentation of some apolipoproteins and arterial proteoglycans and for a pro-inflammatory microenvironment in unstable and much less in stable endarterectomy carotid plaques (Formato et al., 2004). Recently, we evaluated differential protein expression in a considerable number (n=48) of plaques obtained from carotid endarterectomy classified by immunohistochemistry in stable and unstable (Lepedda et al., 2009). Our study was carried out on the premise that plaque stability/instability is associated with distinct patterns of protein expression. We analysed extracts from finely minced tissues in order to allow enrichment in both topically expressed and filtered/retained proteins. A total of 57 distinct spots corresponding to 33 different proteins were identified in both stable and unstable plaques by peptide mass fingerprinting (PMF) analysis, most of which were of plasma origin (about 70%). This suggested the existence of an impaired endothelial barrier function independent from plaque typology. Compared to stable plaques, unstable ones showed reduced abundance of protective enzymes superoxide dismutase 3 and glutathione S-transferase, small HSP 27 and 20, annexin A10, and Rho GDP-dissociation inhibitor and a higher abundance of ferritin light subunit, superoxide dismutase 2 and fibrinogen fragment D. These proteins are described to play a role in either oxidative or inflammatory processes and in the formation and progression of the atherosclerotic plaque. Our proteomic approach, trying to differentiate unstable from stable human carotid plaques, identified, in the former, a panel of proteins with pro-oxidant and pro-inflammatory potentials according to our current understanding of the molecular basis of the atherosclerotic process.

To overcome inter-individual variations in protein expression, Olson et al. applied 2-D differential in gel electrophoresis (2D DIGE) in combination with MS/MS to compare protein distribution in 10 complicated segments located in the internal carotid artery (ICA) with that in 10 more stable segments in the common carotid artery (CCA) from the same patient (Olson et al., 2010). In this way, they identified 19 proteins with differential distribution between ICA and CCA segments. To overcome the problem of plaque heterogeneity, Terzuoli et al. proposed a method for selecting proteins exclusive to plaque by constructing a reference synthetic gel (Terzuoli et al., 2007; Porcelli et al., 2010). This gel, obtained by averaging the positions, shapes and optical densities of spots in 2DE maps from 10 carotid plaque samples was compared with an equivalent synthetic gel constructed using 10 plasma samples from the same carotid surgery patients. The comparison allowed discriminating between plasma and plaque proteins, the latter being potential markers of plaque vulnerability.

Some alternative proteomic approaches have been applied to date in the search of new biomarkers of the atherosclerotic process (table 2).

Human tissues (Methods)	Results	Known functions	Ref.
12 carotid endarterectomy specimens vs 7 non-atherosclerotic mammary arteries (Western array (823 Abs), WB, rt-PCR, IH)	↓↓ apoptosis-linked gene 2 ↑↑ Thrombospondin-2, Mn superoxide dismutase, apolipoprotein B-100, protein-tyrosine phosphatase 1C, apolipoprotein E ↓↓ glycogen synthase kinase-3β	mediator of apoptosis	Martinet et al., 2003
4 pooled unstable carotid plaques vs 4 pooled stable carotid plaques (protein microarray analysis of the expression of 512 proteins)	↑ 21 proteins ↓ 3 proteins	modulation of inflammatory, angiogenic, proliferative, and apoptotic pathways	Slevin et al., 2006
Histological sections from 35 coronary vessels in paraffin or frozen blocks (direct tissue proteomics AQUA methodology)	806 unique proteins identified with high confidence		Bagnato et al., 2007
Carotid plaques from 80 patients that had a secondary cardiovascular event vs 80 sex and age matched event-free patients (during a 3-year follow-up) (LC MS/MS)	Strong positive association between osteopontin and the occurrence of new vascular complications		de Kleijn et al., 2010

↑, increase. ↓, decrease.

Table 2. Alternative approaches in proteomics of the atherosclerotic plaque.

High-throughput western blot analysis, also called western array, was used to screen cell lysates from 12 carotid endarterectomy specimens and 7 non-atherosclerotic mammary arteries, obtained during bypass surgery, with 823 monoclonal antibodies mainly directed against signal-transducing proteins (Martinet et al., 2003). Western arrays showed a highly reproducible pattern of protein expression but also a high rate of false-positive signals (differential protein expression of only 7 of the 15 proteins detected by using this method was confirmed by standard immunoblot assay). A strong down regulation of apoptosis-linked gene 2 (ALG-2) was found, suggesting a novel mechanism inhibiting cell death in human advanced atherosclerotic plaques.